

Cutting Edge: LPS-Induced Emergency Myelopoiesis Depends on TLR4-Expressing Nonhematopoietic Cells

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Systemic bacterial infection is rapidly recognized as an emergency state leading to neutrophil release into the circulation and increased myeloid cell production within the bone marrow. However, the mechanisms of sensing infection and subsequent translation into emergency myelopoiesis have not been defined. In this study, we demonstrate *in vivo* in mice that, surprisingly, selective TLR4 expression within the hematopoietic compartment fails to induce LPS-driven emergency myelopoiesis. In contrast, TLR4-expressing nonhematopoietic cells are indispensable for LPS-induced, G-CSF-mediated myelopoietic responses. Furthermore, LPS-induced emergency myelopoiesis is independent of intact IL-1RI signaling and, thus, does not require inflammatory activation. Collectively, our findings reveal a key and nonredundant role for nonhematopoietic compartment pathogen sensing that is subsequently translated into cytokine release for enhanced, demand-adapted myeloid cell production. *The Journal of Immunology*, 2012, 188: 5824–5828.

Emergency myelopoiesis caused by systemic bacterial infection is characterized by mobilization of neutrophils from the bone marrow (BM), leading to leukocytosis and neutrophilia. In parallel, BM granulocytic precursors increase in frequency and accelerate cell cycle progression to replenish mature, rapidly consumed neutrophils. These processes are governed by the concerted action of multiple hematopoietic growth factors (1–5). However, it is unclear how increased hematopoietic growth factor availability and the induction of emergency myelopoiesis upon systemic pathogen spread are regulated.

Sensing of conserved pathogen-associated molecular patterns occurs through pattern-recognition receptors, such as

TLRs (6). In addition to their expression and function on mature hematopoietic cells, such as macrophages and dendritic cells, TLRs are expressed on immature hematopoietic stem cells, as well as on myeloid (7), lymphoid (8), and dendritic cell (9, 10) progenitor cells, and TLR triggering on hematopoietic stem and progenitor cells can lead to differentiation into mature effector cells (11–14). Importantly, TLR expression on some nonhematopoietic tissues, such as endothelial cells and stromal cells, and their involvement in local immune responses were also demonstrated recently (15–17).

In this study, we addressed the fundamental question of whether sensing of Gram-negative infection-derived LPS and subsequent acute initiation of emergency myelopoiesis depend on TLR expression within the hematopoietic or nonhematopoietic cellular compartment.

Materials and Methods

Mice and generation of reciprocal chimeras

Six- to ten-week-old female wild-type (WT) B6.SJL-Ptprca^aPep3^b/BoyJ (CD45.1⁺) or C57BL/6 TLR4^{-/-} (CD45.2⁺) (18) mice were lethally irradiated with 13 Gy and transplanted *i.v.* with 3–5 × 10⁴ sorted Lin⁻c-Kit^{high} cells of the respective, indicated genotype. All mice were maintained at the Institute for Research in Biomedicine and University Hospital Zurich animal facility and treated in accordance with guidelines of the Swiss Federal Veterinary Office. Experiments were approved by the Dipartimento della Sanità e Socialità, Ticino, Switzerland and Veterinäramt des Kantons Zurich, Switzerland.

Treatment and analysis of mice

WT, TLR4^{-/-}, IL-1RI^{-/-} (19), and chimeric mice were injected twice *i.p.* with 35 µg LPS (*Escherichia coli* 0111:B4, Ultrapure; InvivoGen, San Diego, CA) in a 48-h interval and analyzed 24 h later. Cytospins from peripheral blood (PB) were prepared, and May–Grunwald–Giemsa staining was performed. For FACS analysis of myelopoietic responses, Gr-1 (RB6-8C5) and CD11b (M1/70) Abs were used (both from eBioscience). Myeloerythroid progenitor FACS analysis and CFU assays were performed, as described (7).

WT and TLR4^{-/-} mice were injected *i.p.* with 250 µg/kg body weight human G-CSF (Filgrastim; Amgen) six times in a 12-h interval and analyzed with the same method used for the LPS-injected mice. Plasma G-CSF was analyzed by ELISA, according to the manufacturer's instructions (R&D

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Abbreviations used in this article: BM, bone marrow; PB, peripheral blood; WT, wild-type.

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Systems). For macrophage depletion, mice received 400 μ l liposomal clodronate i.p. (provided by N.v.R) twice in a 48-h interval (for experimental details see Supplemental Fig. 2E).

Tissue macrophage isolation and analysis

Single-cell suspensions from BM and spleen were prepared and subjected to digestion with collagenase D (0.25%; Roche Diagnostics) and DNase I (20 U/ml; Roche Diagnostics). Hepatic macrophages were isolated, as previously reported (20). Peritoneal macrophages were isolated by repeatedly instilling

and aspirating 10 ml warm PBS into the peritoneal cavity. FcRs were blocked using purified CD16/32 (93) Ab and stained with Pacific blue-conjugated CD45.1 (A20), FITC-conjugated CD45.2 (104), PE-conjugated F4/80 (BM8), and allophycocyanin-conjugated CD11b (M1/70) Abs (all from eBioscience) to determine donor/recipient chimerism.

Lung tissues were embedded in Tissue-Tek OCT compound (Sakura), frozen in liquid nitrogen, and stored at -80°C . Five-micron-thick cryosections were cut on a Leica CM 1950 Cryostat (Leica) and fixed in acetone. The following Abs were used: CD45.1-Alexa Fluor 488 (A20), CD45.2-allophycocyanin (104) (both from eBioscience), and CD68 (Serotec) com-

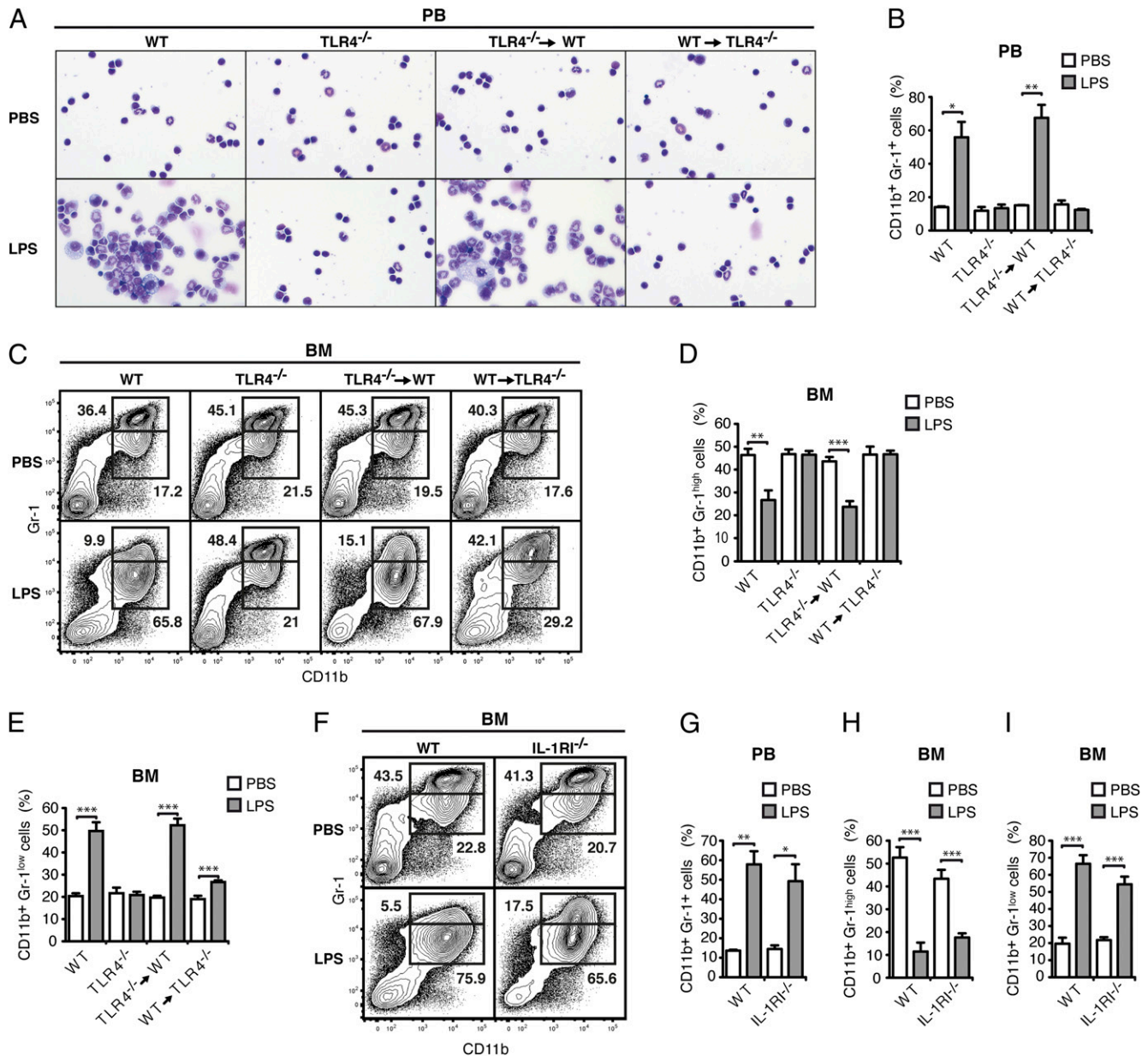


FIGURE 1. PB neutrophilia and BM emergency myelopoiesis are exclusively dependent on TLR4-expressing nonhematopoietic cells in vivo but do not require intact IL-1R signaling. **(A)** May-Grunwald-Giemsa staining of cytopsin preparations of PB from WT, TLR4^{-/-}, and chimeric mice at 72 h after double PBS injection (*top panel*) or LPS (35 μ g from *E. coli* strain 0111:B4) injection (*bottom panel*). Original magnification $\times 250$. **(B)** Percentages of CD11b⁺Gr-1⁺ mature and immature myeloid cells in PB of PBS- or LPS-injected WT, TLR4^{-/-}, and chimeric mice, as described in (A). Mean \pm SEM of two independent experiments, with a total of three PBS- and four LPS-treated mice/group, are shown. **(C)** Representative FACS profile of CD11b⁺Gr-1^{high} and CD11b⁺Gr-1^{low} cells in the BM of WT, TLR4^{-/-}, and chimeric mice after PBS or LPS injection, as described in (A). Percentages of CD11b⁺Gr-1^{high} mature (D) and CD11b⁺Gr-1^{low} immature (E) myeloid cells in the BM of PBS- or LPS-treated WT, TLR4^{-/-}, and chimeric mice after LPS stimulation. Mean \pm SEM of five independent experiments, with a total of 5 PBS- and 10 LPS-treated mice/group, are shown. **(F)** Representative FACS profile of CD11b⁺Gr-1^{high} and CD11b⁺Gr-1^{low} cells in the BM of WT and IL-1RI^{-/-} mice after PBS or LPS injection, as described in (A). **(G)** Percentages of CD11b⁺Gr-1⁺ mature and immature myeloid cells in PB from PBS- or LPS-injected WT and IL-1RI^{-/-} mice, as described in (A). Mean \pm SEM of three independent experiments, with a total of five PBS- and eight LPS-treated mice/group, are shown. Percentages of CD11b⁺Gr-1^{high} mature (H) and CD11b⁺Gr-1^{low} immature (I) myeloid cells in the BM of PBS- or LPS-treated WT and IL-1RI^{-/-} mice after LPS stimulation. Mean \pm SEM of three independent experiments, with a total of five PBS- and eight LPS-treated mice/group, are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

binned with a secondary goat anti-rat-Alexa Fluor 594 Ab (Invitrogen). Immunoslect mounting medium (DAPI; Dianova) was used for embedding stained cryosections. Images were acquired on a Leica SP5 confocal microscope (Leica) and analyzed using Leica LAS AF Lite 2.4 software. Paraformaldehyde-fixed and paraffin-embedded liver sections were analyzed using F4/80 mAb (BM8).

Equations and statistical analyses

Significance of differences was analyzed with an ungrouped or grouped two-tailed Student *t* test or one-sample *t* test, where appropriate. All statistical analyses were calculated with GraphPad Software, v5.01 (Prism).

Results and Discussion

We generated BM chimeric mice with TLR4^{-/-} hematopoiesis on a WT nonhematopoietic background (TLR4^{-/-} → WT mice) and WT hematopoiesis on a TLR4^{-/-} nonhematopoietic background (WT → TLR4^{-/-} mice). At 12 wk after transplantation, no differences in engraftment efficiency or lineage reconstitution were observed, and residual host CD45⁺ BM chimerism was <1% (data not shown, Supplemental Fig. 1A). Remaining host cells consisted mostly of long-lived

CD3⁺ T cells (Supplemental Fig. 1B). Importantly, the vast majority (>97%) of tissue-resident macrophages in BM, spleen, lung, and peritoneal cavity were of donor origin 3 mo after transplantation (Supplemental Fig. 1C, 1D). However, ~20% of hepatic macrophages were still host derived, consistent with previous findings (20). Chimeric and control mice were challenged with LPS or PBS and analyzed for the first hallmark of emergency myelopoiesis: induction of neutrophilia and increase in myeloid precursors in the PB. No differences between nonreconstituted (WT and TLR4^{-/-}) and control-reconstituted (WT → WT and TLR4^{-/-} → TLR4^{-/-}) control mice were observed (data not shown). There was an increase in mature and immature neutrophils in the PB of WT and TLR4^{-/-} → WT mice, as revealed by morphological examination of cytospin preparations and by immunophenotypical analysis using CD11b and Gr-1 Abs. In contrast, TLR4^{-/-} and WT → TLR4^{-/-} mice failed to mount a respective response to systemic LPS challenge (Fig. 1A, 1B, Supplemental Fig. 1E).

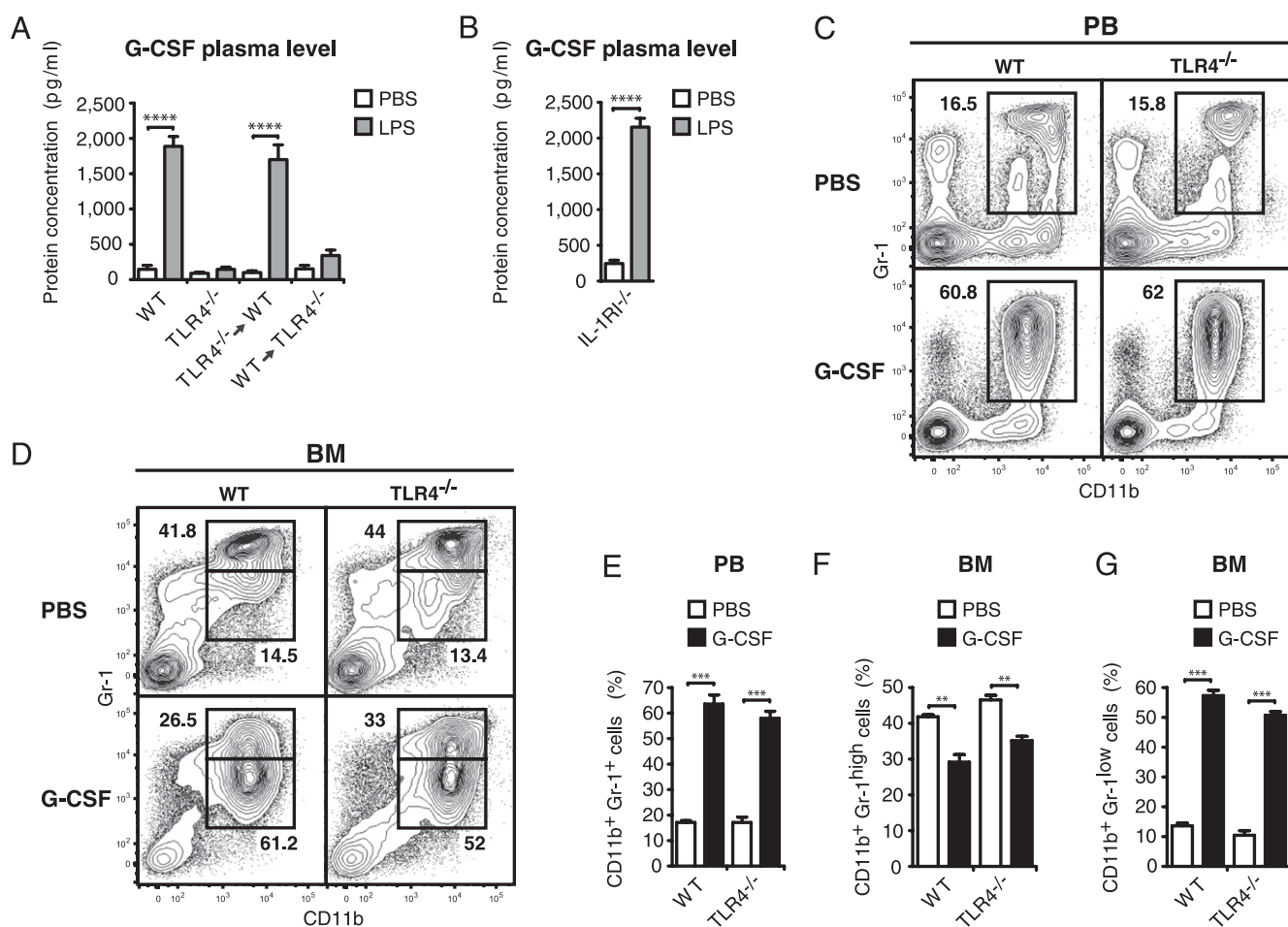


FIGURE 2. G-CSF is strongly increased upon selective stimulation of TLR4-expressing nonhematopoietic cells and is a major mediator of emergency myelopoiesis in vivo. **(A)** Plasma G-CSF protein levels in PBS- or LPS-treated (35 μ g from *E. coli* strain 0111:B4) WT, TLR4^{-/-}, and chimeric mice analyzed 72 h after double injection. Mean \pm SEM of three independent experiments, each with three PBS- and four LPS-treated mice/group, are shown. **(B)** Plasma G-CSF protein levels in PBS- or LPS-treated IL-1RI^{-/-} mice, treated as described in (A). Mean \pm SEM of two independent experiments, with a total of three PBS- and six LPS-treated mice/group, are shown. **(C)** Representative FACS profile showing myeloid cells (CD11b⁺Gr-1⁺) in PB of G-CSF-treated WT mice and TLR4^{-/-} mice. The treatment regimen consisted of six injections with 250 μ g human G-CSF/kg body weight every 12 h over a period of 3 d. Mice were analyzed 24 h after the last injection. **(D)** Representative FACS profile of CD11b⁺Gr-1^{high} and CD11b⁺Gr-1^{low} cells in the BM of WT and TLR4^{-/-} mice following G-CSF injection, as described in (C). **(E)** Percentages of CD11b⁺Gr-1⁺ mature and immature myeloid cells in PB from PBS- or G-CSF-injected WT and TLR4^{-/-} mice, as described in (C). Mean \pm SEM of two independent experiments, with a total of four G-CSF-treated mice, are shown. Percentages of CD11b⁺Gr-1^{high} mature **(F)** and CD11b⁺Gr-1^{low} immature **(G)** myeloid cells in the BM of PBS- or G-CSF-injected WT and TLR4^{-/-} mice, as described in (C). Mean \pm SEM of two independent experiments, with a total of four G-CSF-treated mice, are shown. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

Next, we studied the second hallmark of emergency myelopoiesis: BM myeloid proliferation and differentiation. In WT and TLR4^{-/-} → WT mice, mature myeloid cells (CD11b⁺Gr-1^{high}) decreased significantly upon LPS injection, whereas the frequency and total numbers of immature promyelocytes and myelocytes (CD11b⁺Gr-1^{low}) (21, 22) increased up to 2.5-fold (Fig. 1C–E, data not shown). In contrast, no change in cellular composition could be observed in TLR4^{-/-} or WT → TLR4^{-/-} mice, whereas a small, but still significant relative, but not absolute, increase in immature CD11b⁺Gr-1^{low} cells was detectable in WT → TLR4^{-/-} mice (Fig. 1E, data not shown). Because granulocyte-macrophage progenitors and CFU-granulocyte increased in WT and both TLR4^{-/-} → WT and WT → TLR4^{-/-} mice, signaling through TLR4 in hematopoietic and nonhematopoietic cells can skew early hematopoiesis toward myeloid differentiation (Supplemental Fig. 2A–C). However, nonhematopoietic cell-expressed TLR4 is sufficient, as well as necessary, for induction of full-blown emergency myelopoiesis.

IL-1β is a paradigmatic proinflammatory cytokine driving the expression of various myelopoiesis-acting cytokines, such as *G-CSF*, *M-CSF*, and *Gm-CSF* (23). Thus, initiation of emergency myelopoiesis may involve activation of the inflammasome, leading to IL-1β release that stimulates IL-1RI-expressing cells to secrete myelopoiesis-acting growth factors, which, in turn, promote emergency myelopoiesis, as suggested recently (24). However, LPS stimulation caused an identical emergency myelopoietic response in IL-1RI^{-/-} mice compared with WT mice (Fig. 1F–I, data not shown). Thus, we conclude that IL-1RI signaling may be involved in alternative pathways leading to reactive neutrophilia, such as treatment with the adjuvant alum (22, 24). However, our findings unambiguously show that LPS-induced emergency myelopoiesis does not require intact IL-1RI signaling, a clinically important finding with respect to current anti-IL-1-directed therapies (25).

We next sought to determine the mediating signal, which is released upon LPS-induced TLR4 signaling in nonhematopoietic cells. To this end, we focused on the key myeloid cytokine G-CSF. Although G-CSF is not indispensable for emergency myelopoiesis, G-CSF^{-/-} mice show markedly reduced and delayed kinetics of neutrophilia (26, 27). Moreover, G-CSF is successfully applied to treat congenital or chemotherapy-induced neutropenia (1) and, most importantly, is highly increased in patient serum during bacterial-induced inflammation or sepsis (28, 29). In accordance with these clinical findings, a significant ~10-fold increase in plasma G-CSF levels was observed after LPS injection in WT and TLR4^{-/-} → WT mice, whereas no significant increase was detectable in WT → TLR4^{-/-} mice (Fig. 2A). An identical increase in G-CSF plasma levels was observed in IL-1RI^{-/-} mice (Fig. 2B). The minor, nonsignificant increase in G-CSF in WT → TLR4^{-/-} mice might be responsible for the small relative increase in immature BM CD11b⁺Gr-1^{low} cells in these mice (Fig. 1E) and is possibly mediated via hematopoietic cell (i.e., progenitor cell or myeloid cell) LPS sensing. However, it is insufficient to generate a sustained myelopoietic response. We also assessed plasma levels of M-CSF, GM-CSF, SCF, IL-3, and FLT3L. Of these, only FLT3L levels increased significantly, specifically in WT and WT → TLR4^{-/-} mice, indicating a primarily hematopoietic cell-mediated induction or origin upon LPS stimulation (data not shown).

To determine whether the massive increase in G-CSF upon LPS stimulation is, as a single cytokine, sufficient to translate into emergency myelopoiesis, we injected human G-CSF into WT and TLR4^{-/-} mice. This treatment led to qualitative and quantitative responses that were indistinguishable from those induced by LPS application (Fig. 2C–G, data not shown).

Thus, our data reveal that the primary, indispensable *in vivo* sensing site for LPS-induced emergency myelopoiesis is a TLR4-expressing nonhematopoietic cellular compartment that translates this signal into massive G-CSF release. We reason that the few remaining primarily hepatic macrophages do not play a major role in this context, because macrophage-depleted mice show a similar myeloid response as do control mice (Supplemental Fig. 2D–H). Future studies are needed to clarify the identity and localization of the TLR4-expressing and, likely cytokine-releasing, nonhematopoietic parenchymal or stromal cell populations. Selective delivery of TLR mimetics to these cells could then be an intriguing therapeutic possibility to enhance myeloid cell regeneration, supplementing the current need for application of single recombinant cytokines.

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Disclosures

The authors have no financial conflicts of interest.

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